

# Microwell Hybridization Assay for Detection of PCR Products from *Mycobacterium tuberculosis* Complex and the Recombinant *Mycobacterium smegmatis* Strain 1008 Used as an Internal Control

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**A microwell hybridization assay was developed for the detection of the PCR products from both *Mycobacterium tuberculosis* complex bacteria and the recombinant *Mycobacterium smegmatis* strain 1008 that is used as an internal control to monitor inhibition in the PCR based on the *M. tuberculosis* complex-specific insertion sequence IS6110. The test is based on specific detection with digoxigenin-labeled oligonucleotide probes of biotinylated PCR products which are captured in a microtiter plate coated with streptavidin. The captured PCR products are hybridized separately with two probes, one specific for the PCR product from IS6110 from *M. tuberculosis* complex and the other specific for the PCR fragment from the modified IS6110 fragment from the recombinant *M. smegmatis* 1008. The microwell hybridization assay discriminates perfectly between the two types of amplicon. The amount of PCR product that can be detected by this assay is 10 times less than that which can be detected by agarose gel electrophoresis. The test can be performed in 2 h. It is much faster and less laborious than Southern blot hybridization. Furthermore, the interpretation of results is objective. The assay was used with 172 clinical samples in a routine microbiology laboratory, and the results were in complete agreement with those of agarose gel electrophoresis and Southern blot hybridization.**

PCR is a very rapid and sensitive technique for the detection of *Mycobacterium tuberculosis* DNA in clinical samples (2, 4–7, 10–14, 17). Our PCR assay for the detection of *M. tuberculosis* complex bacteria is based on the *M. tuberculosis* complex-specific insertion sequence IS6110 (10–12). Recombinant *Mycobacterium smegmatis* 1008 containing a modified IS6110 fragment is used as an internal control to monitor individual samples for PCR inhibition (9). Amplification of the modified IS6110 fragment results in a PCR product which is 56 bp longer than the *M. tuberculosis* IS6110 PCR fragment. Agarose gel electrophoresis and Southern blot hybridization with an internal probe are usually used for the detection and analysis of PCR products from both *M. tuberculosis* complex and the modified IS6110 fragments (10, 14). Successful large-scale application of this PCR in a routine microbiology laboratory has been described previously (14).

However, hybridization procedures on membranes are laborious and the readings are subjective, and therefore we developed an amplicon detection method which is quicker and simpler to perform. In our test, one of the strands of the PCR product is labeled with biotin by incorporation of a biotinylated primer. The biotinylated strand is captured in the microwells of a streptavidin-coated microtiter plate. In our procedure, the hybridization is done with digoxigenin (DIG)-labeled oligonucleotide probes specific for the IS6110 amplicons from *M. tuberculosis* complex or specific for the modified IS6110 amplicons from the *M. smegmatis* 1008 internal control.

## MATERIALS AND METHODS

**Clinical samples.** Clinical samples from patients suspected of having tuberculosis were obtained from various hospitals in the county of Friesland, in the northern part of The Netherlands. The 172 samples included pleural fluid ( $n = 48$ ), tissue biopsy samples ( $n = 37$ ), sputum ( $n = 30$ ), bronchoalveolar lavage fluid ( $n = 21$ ), pus ( $n = 18$ ), cerebrospinal fluid ( $n = 8$ ), feces ( $n = 6$ ), and urine ( $n = 4$ ). On the day of receipt, the clinical samples were split with appropriate precautions. One portion was used for conventional mycobacterial detection methods (microscopy and culture), and one portion was used for PCR (14) followed by agarose gel electrophoresis with Southern blot hybridization and by microwell hybridization.

**Conventional mycobacterial detection.** Microscopy was performed after Ziehl-Neelsen staining (16). Respiratory samples were decontaminated according to standard procedures (8) and inoculated on Löwenstein-Jensen medium. Non-respiratory samples, after standard pretreatment (8), were inoculated on Löwenstein-Jensen medium and Middlebrook 7H11 medium. The specimens were incubated at 37°C for 2 months and examined weekly for growth.

**DNA extraction from clinical samples.** DNA extraction from clinical samples was performed by the guanidinium thiocyanate-diatoms method as previously described (1, 10, 14). One negative sample to every five clinical samples was included in each series to monitor for cross-contamination during DNA extraction. When a negative control sample gave a positive PCR result, this indicated the potential presence of false positives in that series. The PCR would need to be repeated on the positive clinical samples in that series with another portion of the clinical specimen, if available, or a fresh specimen. The efficiency of DNA extraction was monitored with two sputum samples containing 100 and 1,000 *Mycobacterium bovis* BCG bacteria. When a spiked sputum sample gave a negative PCR result, this indicated that the DNA extraction procedure was inefficient. In that case the DNA extraction and PCR would need to be repeated for all the negative clinical samples in the series.

**PCR.** The PCR was performed with primers INS1 and INS2 as described previously (14). Primer INS2 was biotin labeled at the 5' end of the DNA via a six-carbon spacer arm (Isogen Bioscience, Amsterdam, The Netherlands). For each clinical sample, two PCRs were performed, one with 10 µl of undiluted DNA extract and one with 10 µl of 10-fold diluted DNA extract. All PCRs were spiked with 50 fg (equivalent to 10 bacteria or 10 copies of IS6110) of DNA from recombinant *M. smegmatis* 1008 containing a modified IS6110 fragment used as an internal control for inhibition (9). This internal control was added during the preparation of the reaction mixture. The internal control is amplified with the same efficiency as *M. tuberculosis* DNA. The amount of *M. smegmatis* 1008 DNA

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was chosen in such a way that competition between both targets could not result in false-negative results, since we have shown that competition only leads to false-negative results for the *M. tuberculosis* IS6110 target when there is a >100-fold excess of the internal control *M. smegmatis* 1008 DNA (9). When the PCR of a clinical sample was negative for the amplicons from both *M. smegmatis* 1008 and *M. tuberculosis*, then inhibitors were present and the DNA extraction procedure would have to be repeated.

Three samples containing 250, 25, and 2.5 fg of DNA from *M. tuberculosis* 1 with eight copies of IS6110 per genome (10) (equivalent to 50, 5, and 0.5 bacteria or 400, 40, and 4 copies of IS6110, respectively) and one sample without DNA were tested in each run, to monitor the performance of the amplification reaction. When the *M. tuberculosis* DNA controls were negative, that indicated a failure in the PCR mixture or amplification procedure, so only the PCR would need to be repeated. When the sample which contained no DNA was positive in PCR, that indicated contamination. The PCR procedure would need to be repeated.

**Agarose gel electrophoresis.** Electrophoresis of the PCR products (10  $\mu$ l) was performed on 2% agarose gels stained with ethidium bromide. PCR products from *M. tuberculosis* are 245 bp, and PCR products from the *M. smegmatis* strain 1008 containing a modified IS6110 fragment are 301 bp.

**Southern blot hybridization.** The Southern blot hybridization assay was performed with an internal 188-bp DIG-labeled probe as described previously (10).

**Microwell hybridization assay.** Twenty microliters of the PCR product was diluted in 200  $\mu$ l of 1 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.5% Tween 20. Two wells of a streptavidin-coated microtiter plate (high capacity; Boehringer, Mannheim, Germany) were each filled with 100  $\mu$ l of the diluted PCR products. The biotin binding capacity of the wells is 50 times higher than the maximum number of molecules that can be formed in a total PCR. Capture was allowed to proceed for 30 min at 37°C, and then the wells were washed four times with 350  $\mu$ l of 1 $\times$  SSC in a microtiter plate washer at room temperature. The captured biotinylated PCR products were denatured by adding 100  $\mu$ l of 0.1 M NaOH to each well. Nonbiotinylated DNA strands were removed by washing twice with 1 $\times$  SSC at room temperature. To each well was added 100  $\mu$ l of hybridization mix (1 $\times$  SSC, 0.05% Tween 20, 0.05% bovine serum albumin, 50% formamide) containing 2 pmol of *M. tuberculosis* complex IS6110-specific probe TB245 (5' DIG-GGTTCGCCTACGTGGCC 3') or 2 pmol of recombinant *M. smegmatis* internal control IS6110 probe MSM301 (5' DIG-CATTGCGCCGGCATGTAGCA 3') (Isogen Bioscience). After hybridization for 30 min at room temperature, the wells were washed twice with 2 $\times$  SSC–0.5% sodium dodecyl sulfate and then twice with 2 $\times$  SSC. To each well, 100  $\mu$ l of anti-DIG-peroxidase (750 U/ml; Boehringer) diluted 1/1,000 in universal conjugate buffer (Boehringer) was added. Plates were incubated for 30 min at 37°C, and the wells were washed with phosphate-buffered saline–0.05% Tween 20 and 100  $\mu$ l of tetramethylbenzidine substrate (TMB blue; Boehringer). The wells were incubated for 10 min at room temperature, and the reaction was stopped by adding 100  $\mu$ l of 2.5 M H<sub>2</sub>SO<sub>4</sub>. The  $A_{450}$  was read with a microtiter plate reader. Positive hybridization controls were included in each microwell hybridization assay. They consisted of standard solutions of 10- and 100-fold dilutions of PCR products derived from 1 pg of *M. tuberculosis* 1 DNA and 10 pg of *M. smegmatis* 1008 DNA, respectively. Water was used as a negative control.

## RESULTS

**Validation of the microwell hybridization assay.** Table 1 shows the results of 17 microwell hybridization assays done with the amplicon solutions which form the positive controls. These assays were performed on different days. Probe TB245 hybridized with the *M. tuberculosis* amplicons but not with the recombinant *M. smegmatis* amplicons. Probe MSM301 hybridized only with the *M. smegmatis* amplicons. The  $A_{450}$  values of the *M. smegmatis* amplicons after hybridization with probe TB245 and those of *M. tuberculosis* amplicons with probe MSM301 were the same as those for hybridization in wells in which amplicons were absent.

When the PCR product from 1 pg of *M. tuberculosis* DNA was used, 0.1  $\mu$ l of the PCR product (10  $\mu$ l of a 1:100 dilution) could be detected in the microwell hybridization assay (Table 1) whereas by agarose gel electrophoresis only 1  $\mu$ l of PCR product (10  $\mu$ l of a 1:10 dilution) could be detected (results of agarose gel electrophoresis not shown). The same is true for the PCR product from 10 pg of *M. smegmatis* 1008 DNA.

**Interpretation of absorbance values in the microwell hybridization assay.** In these 17 microwell hybridization assays, the  $A_{450}$  with probe TB245 of the PCR products of 19 negative sputum samples that were used to control for cross-contamination was  $0.1 \pm 0.03$  (mean  $\pm$  standard deviation). This value

TABLE 1. Validation of the microwell hybridization assay<sup>a</sup>

Input in PCR	Dilution of PCR product <sup>b</sup>	Probe <sup>c</sup>	Mean (SD) $A_{450}$
<i>M. tuberculosis</i> 1 DNA (1 pg) (1,600 copies of IS6110)	1:10	TB245	1.66 (0.61)
	1:100	MSM301	0.08 (0.02)
	1:10	TB245	0.32 (0.14)
	1:100	MSM301	0.09 (0.02)
<i>M. smegmatis</i> 1008 DNA (10 pg) (2,000 copies of IS6110)	1:10	TB245	0.08 (0.02)
	1:100	MSM301	1.92 (0.80)
	1:10	TB245	0.08 (0.02)
	1:100	MSM301	0.46 (0.17)
Water		TB245	0.08 (0.02)
		MSM301	0.08 (0.02)

<sup>a</sup> A total of 17 separate tests were done on different days.

<sup>b</sup> Total volume of PCR product in each assay, 10  $\mu$ l.

<sup>c</sup> TB245, probe specific for IS6110 amplicons from *M. tuberculosis* complex; MSM301, probe specific for modified IS6110 amplicons from recombinant *M. smegmatis* 1008.

is comparable to that of water (Table 1). The cutoff value for PCR positivity was set at 0.2 (mean plus three standard deviations).

When a 301-bp PCR product was identified by agarose gel electrophoresis, this indicated that the sample was free of inhibitors. The cutoff value for inhibition in the microwell hybridization assay was determined with the PCR products from the 172 clinical samples that gave only a 301-bp fragment on agarose gel. The  $A_{450}$  of these 257 PCR products with probe MSM301 in the microwell hybridization assay was  $2.4 \pm 0.7$  (mean  $\pm$  standard deviation). The cutoff value for inhibition was set at 1.0 (mean minus two standard deviations).

Table 2 shows how the results of the microwell hybridization assay with clinical samples should be interpreted. When the  $A_{450}$  with probe TB245 is >0.2, the sample contains *M. tuberculosis* complex DNA. However, when the  $A_{450}$  with this probe is  $\leq 0.2$ , the results with probe MSM301 are needed to allow evaluation of the results. When the  $A_{450}$  with probe MSM301 is <1.0, *M. tuberculosis* complex DNA is absent, but when the  $A_{450}$  with probe MSM301 is  $\leq 1.0$ , inhibitors are present. In this case, the result of the 10-times-diluted DNA extract from the sample should be assessed. When the 10-times-diluted DNA extract still contains inhibitors, the DNA purification procedure should be repeated.

**Comparison of results of agarose gel electrophoresis, Southern blot hybridization, and microwell hybridization.** Figure 1 and Table 3 show the results of the three methods of amplicon

TABLE 2. Interpretation of absorbance values in the microwell hybridization assay

$A_{450}$ with <i>M. tuberculosis</i> complex probe TB245	$A_{450}$ with <i>M. smegmatis</i> 1008 probe MSM301	Interpretation of assay
>0.2	— <sup>a</sup>	Positive for <i>M. tuberculosis</i> complex
$\leq 0.2$	>1.0 <sup>b</sup>	Negative for <i>M. tuberculosis</i> complex
$\leq 0.2$	$\leq 1.0$	Inhibition <sup>c</sup> in PCR

<sup>a</sup> —, not relevant since the *M. tuberculosis* complex signal is positive.

<sup>b</sup> No inhibition in the PCR.

<sup>c</sup> When there is inhibition in the PCR, the results of the 10-times-diluted DNA extract should be interpreted.

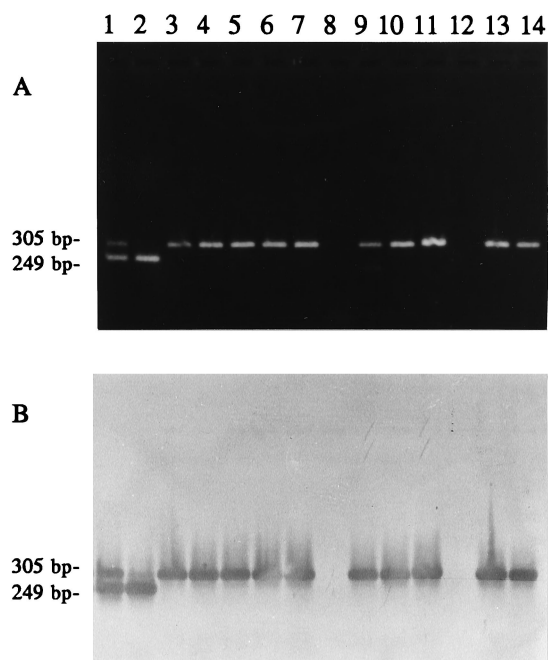


FIG. 1. Comparison of results of agarose gel electrophoresis (A) and Southern blot hybridization (B) with PCR products from 14 clinical samples. For details see Table 3.

detection—microwell hybridization, agarose gel electrophoresis, and Southern blot hybridization—for 14 PCR products derived from different clinical samples. There was complete agreement among the results of all three amplicon detection methods. Two samples were positive, in two samples there was inhibition, and the remaining samples were negative.

When 172 clinical samples were tested, the results of the microwell hybridization assay were in complete agreement with those of agarose gel electrophoresis and Southern blot

hybridization (data not shown). It should be noted that these tests were done in a blinded fashion; the results of the microwell hybridization assay were not known when the agarose gel electrophoresis and Southern blot hybridization were done and vice versa. The  $A_{450}$  values after hybridization with probe TB245 for samples containing *M. tuberculosis* were always greater than 5 times the cutoff value.

**Results of PCR after microwell hybridization.** We found 21 *M. tuberculosis* complex PCR-positive samples and 148 PCR-negative samples in the microwell hybridization assay. DNA extraction and PCR procedures met the required standards. The PCR results corresponded with the bacteriological (culture and smear) and clinical findings (data not shown). For three samples no conclusions could be drawn since the undiluted and the 10-times-diluted samples contained inhibitors. Another DNA purification procedure was then done on these three samples, and the PCR products were tested by agarose gel electrophoresis and Southern blot hybridization. The samples were free of inhibitors and contained no *M. tuberculosis* DNA. Of the 21 *M. tuberculosis* PCR-positive samples, 5 (24%) were positive when the undiluted and 10-times-diluted samples were tested, 6 (29%) were positive only when the undiluted samples were tested, indicating that only a few *M. tuberculosis* complex bacteria were present in the sample, and 10 (48%) were positive only when the 10-times-diluted DNA extract from the sample was tested, indicating the presence of inhibitors. Of the 148 negative samples, 109 (74%) were free of inhibitors when tested undiluted and the remaining 39 (26%) were free of inhibitors when the 10-times-diluted samples were tested.

## DISCUSSION

We developed a microwell hybridization assay to simplify the detection of *M. tuberculosis* complex and modified *M. smegmatis* IS6110 PCR fragments. The test uses biotinylated primers in the PCR, resulting in biotinylated PCR products. These products are captured in a microtiter plate with streptavidin-coated wells and detected by hybridization with DIG-labeled

TABLE 3. Comparison of microwell hybridization with agarose gel electrophoresis and Southern blot hybridization

Sample no. <sup>a</sup>	Microwell hybridization		Interpretation of assay <sup>c</sup>	Agarose gel electrophoresis		Interpretation of assay	Southern blot hybridization		Interpretation of assay
	<i>A</i> <sub>450</sub> with <sup>b</sup> :			Presence of fragment in gel <sup>d</sup>			Presence of fragment on blot		
	TB245	MSM301		245 bp	301 bp		245 bp	301 bp	
1	1.894	2.783	Positive	+	+	Positive	+	+	Positive
2	2.462	0.937	Positive	+	—	Positive	+	+/—	Positive
3	0.129	2.395	Negative	—	+	Negative	—	+	Negative
4	0.123	2.458	Negative	—	+	Negative	—	+	Negative
5	0.125	2.264	Negative	—	+	Negative	—	+	Negative
6	0.161	2.185	Negative	—	+	Negative	—	+	Negative
7	0.161	2.458	Negative	—	+	Negative	—	+	Negative
8	0.076	0.083	Inhibition	—	—	Inhibition	—	—	Inhibition
9	0.112	2.304	Negative	—	+	Negative	—	+	Negative
10	0.134	2.052	Negative	—	+	Negative	—	+	Negative
11	0.152	2.553	Negative	—	+	Negative	—	+	Negative
12	0.083	0.083	Inhibition	—	—	Inhibition	—	—	Inhibition
13	0.122	2.401	Negative	—	+	Negative	—	+	Negative
14	0.131	2.271	Negative	—	+	Negative	—	+	Negative

<sup>a</sup> Visual results of agarose gel electrophoresis and Southern blot hybridization of these samples are shown in Fig. 1.

<sup>b</sup> TB245, probe specific for IS6110 amplicons from *M. tuberculosis* complex; MSM301, probe specific for modified IS6110 amplicons from recombinant *M. smegmatis* 1008.

<sup>c</sup> Positive, *M. tuberculosis* complex DNA present; negative, *M. tuberculosis* complex DNA absent; inhibition, inhibitors present.

<sup>d</sup> +, positive signal; +/-, weakly positive signal; —, no signal.

oligonucleotide probes. Others have described similar capture assays for the detection of amplicons (3, 15, 17, 18), but these authors did not use a built-in control for inhibition of the PCRs. In our assay, two DIG-labeled oligonucleotide probes are used, permitting detection of amplicons from the *M. tuberculosis* complex and amplicons from recombinant *M. smegmatis* 1008 (containing a modified IS6110). The latter is used as the internal control in the same PCR. There was no cross-hybridization between IS6110 PCR products from *M. tuberculosis* with the recombinant *M. smegmatis*-specific probe and vice versa.

We have shown that when 172 clinical samples were tested in a routine laboratory, there was complete agreement among the interpretation of the results of microwell hybridization, agarose gel electrophoresis, and Southern blot hybridization. Our microwell hybridization assay is very simple and can be performed within 2 h when one microtiter plate which permits the testing of about 15 clinical samples is used. The amount of PCR product that can be detected by our assay is 10 times less than that which can be detected by agarose gel electrophoresis, and our assay is much faster and less laborious than Southern blot hybridization. Furthermore, the interpretation of results is objective.

We conclude that the microwell hybridization assay is a suitable amplicon detection method for a laboratory performing PCR on clinical samples on a routine basis.

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